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The protocadherin papc is involved in the organization of the epithelium along the segmental border during mouse somitogenesis

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Abstract

The anterior and posterior halves of individual somites adopt distinct fates during somitogenesis, which is crucial for establishing the metameric pattern of axial tissues such as the vertebral column and peripheral nerves. Genetic analyses have demonstrated that the specification of cells to an anterior or posterior fate is intimately related to the process of segmentation. Inactivation of the transcription factor Mesp2, or components of the Notch signaling pathway, led to defects in segmentation and a loss of anterior/posterior polarity. Target genes in mice that could mediate the morphological events associated with segmentation or polarity have not been identified. Studies in *Xenopus* and zebrafish have demonstrated that the protocadherin, *papc*, is expressed in an anterior-specific manner in the presumptive somites of the presomitic mesoderm and is required for normal somitogenesis. Here, we examine the role of *papc* in directing segmentation in the mouse. We demonstrate that papc is expressed in a dynamic pattern within the first two presumptive somites (0 and −1) at the anterior end of the presomitic mesoderm. The domain of *papc* transcription in somite 0 starts broad and becomes progressively restricted to the anterior edge. Transcription in somite −1 over the same time remains broad. Analysis of targeted null mutations revealed that transcription of *papc* is dependent on *Mesp2*. The dynamic nature of *papc* transcription in somite 0 requires the expression of *lunatic fringe*, which modifies the activation of the Notch signaling pathway and is required for proper segmentation of somites. Treatment of embryonic mouse tails in a hanging drop culture with a putative dominant-negative mutation of *papc* disrupted the epithelial organization of cells at the segmental borders between somites. Together, these data indicate that *papc* is an important regulator of somite epithelialization associated with segmentation.

Keywords: Protocadherin; papc; Mesp2; Lunatic fringe; Somitogenesis; Boundary formation; Paraxial Mesoderm

Introduction

The segmental vertebrate body plan is manifested in the metameric pattern of the vertebrae, skeletal muscle, peripheral nerves, and vasculature, which is established early in embryogenesis through the process of somitogenesis. Somites are derived from the paraxial mesoderm, which involutes through the anterior region of the late primitive streak and migrates to form two discrete bands of presomitic mesoderm, which flank the neural tube (reviewed in Christ and Ordhal, 1995). Each somite will give rise to axial skeleton (vertebrae and ribs), skeletal muscle, and dermis. In addition, the somites impose a segmental pattern on the vasculature and peripheral nerves. Somites form in a cyclic manner that leads to cells located at the anterior end presomitic mesoderm pinching off and forming epithelial balls (Christ and Ordahl, 1995; Christ and Wilting, 1992; Tam and Traynor, 1994). Associated with this process, the mesenchymal cells at the anterior end of the presomitic mesoderm undergo an increase in number, density, and adhesion, followed by the formation of a distinct segmental boundary

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(for review, see Keynes and Stern, 1988; Tam and Trainor, 1994). The majority of the genes that are known to regulate somitogenesis are transcription factors or receptors that activate transcription factors. Little is known about the genes that mediate the morphological events associated with somite formation; therefore, identifying these downstream target genes is of central importance to our understanding of the regulation of segmentation in vertebrates.

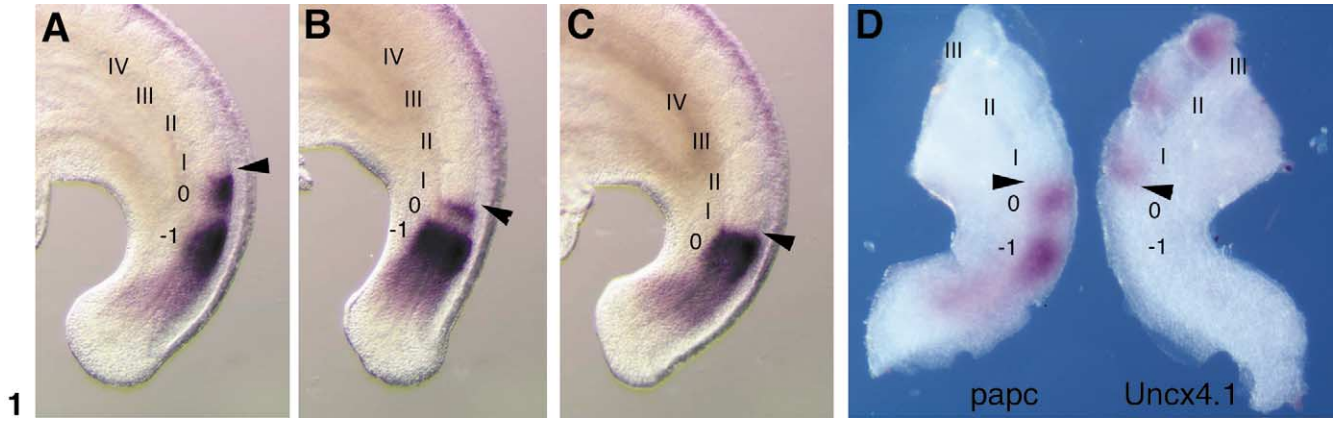
The temporal control of somitogenesis is dependent on an intrinsic segmentation clock embedded in the presomitic mesoderm. The clock is made evident by the oscillation of *HES1, 2, 7* and *lunatic fringe (L-Fng)* transcription along the length of the presomitic mesoderm at a frequency consistent with the time required for the formation of a single somite (Aulehla and Johnson, 1999; Bessho et al., 2001; Jouve et al., 2000; McGrew et al., 1998; Palmeirim et al., 1997). Temporal periodicity is then translated into spatial periodicity through the influence of L-Fng on the Notch signaling pathway at the anterior end of the presomitic mesoderm (Barrantes et al., 1999; Evrard et al., 1998; McGrew et al., 1998; Zhang and Gridley, 1998). Targeted null mutations in *Notch1,* its ligands *Dll1* and *Dll3,* and *Presenilin1,* a gene that modifies Notch activity, disrupt the timing of somite formation which impacts the somite size and registry across the neural tube (Conlon et al., 1995; Hrabe de Angelis et al., 1997; Kusumi et al., 1998; Shen et al., 1997; Swiatek et al., 1994; Wong et al., 1997). However, somitogenesis is not completely abolished in these mutants, suggesting that additional regulation is required for generating somite periodicity.

In addition to the formation of segmental boundaries, the subdivision of individual somites into anterior and posterior halves is important for the establishment of the segmental organization of the vertebrae and peripheral nerves (Goldstein and Kalcheim, 1992). Specification of somitic cells to an anterior or posterior fate is established in the presomitic mesoderm prior to overt somitogenesis (Aoyama and Asamoto, 1988; Bronner-Fraser and Stern, 1991). Anterior/posterior (A/P) polarity is manifested genetically by the restricted expression of signaling factors (e.g., FGFR1, EphA4, and ephrinB2), transcription factors (e.g., Uncx4.1, Pax-1, and Pax-9), and extracellular matrix proteins (e.g., collagen IX) to the anterior or posterior halves of the newly formed somites (reviewed in Rawls et al., 2000). Specification of A/P polarity is regulated through active Notch signaling. Notch ligands *Dll1* and *Jag1* are coexpressed in the posterior half of somite I and the forming somite (0), while *Dll3* is expressed in the anterior halves of both somites (Barrantes et al., 1999; Dunwoodie et al., 1997; Mitsiadis et al., 1997; Zhang and Gridley, 1998). The juxtaposition of *Dll1*/*JagI* and *Dll3* across the forming somite boundary, as well as within the forming somite, directs differential responses of Notch in these two domains. Consistent with this, null mutations in *Dll1* and *Dll3* resulted in a disruption of anterior- and posterior-specific gene expression, fusion of the vertebrate, and a failure of the peripheral nerves to develop a proper segmental pattern (Barrantes et al., 1999; Dunwoodie et al., 2002; Hrabe de Angelis et al., 1997). The specification of the posterior half of the somite by Notch is also dependent on the expression of *Presenilin1,* a gamma-secretase required for the intramembranous cleavage of the intracellular domain of ligand-activated Notch (Koizumi et al., 2001). This suggests that the regulation of specification by Notch occurs by two different mechanisms in the presumptive somite.

Specification of the anterior half of the somite also requires the expression of *Mesp2,* a basic helix-loop-helix (bHLH) transcription factor (Saga et al., 1997; Takahashi et al., 2000). In *Mesp2*$^{-/-}$ embryos, anterior-specific transcription is lost in the presomitic mesoderm and somites (Saga et al., 1997; Takahashi et al., 2000). Posterior- specific genes are expressed in the anterior halves of the somites from this mutant. The vertebrae that formed in the *Mesp2*$^{-/-}$ embryos have a duplication of the posterior structures, suggesting that the somite becomes posteriorized in the absence of *Mesp2. Mesp2* is transcribed in a broad domain that encompasses presumptive somite -1 and becomes restricted to the anterior half of the presumptive somite -1 prior to the formation of the newest somite. The restriction of transcription fails to occur in *Presenilin1*$^{-/-}$ embryos, suggesting that Notch signaling is responsible for reducing *Mesp2* transcription in the posterior domain (Koizumi et al., 2001). However, the polarity defects associated with the *Mesp2* null mutants can be rescued by activated *Notch,* suggesting that *Notch* lies downstream of *Mesp2* in the specification of the anterior fate (Takahashi et al., 2000). These studies reveal a complex relationship between *Mesp2* and *Notch* during the specification of A/P polarity.

In addition to specifying spatial identity, there is another layer of regulation that maintains the boundary between the anterior and the posterior halves of the somite. The initiation of somitogenesis is associated with an increase in the cell-adhesion properties of cells in the anterior presomitic mesoderm (Cheney and Lash, 1984). It has been demonstrated that *paraxis,* a bHLH transcription factor, is essential for the restriction of posterior-specific transcription in the newly formed somite (Johnson et al., 2001). Paraxis is also required for the epithelialization of the somite (Burgess et al., 1996), raising the possibility that the increase in cell adhesion associated with the mesenchyme to epithelium transition may play a role in maintaining the intrasomitic boundary.

Genetic and tissue aggregation studies have demonstrated that Ca^{2+}-dependent cadherin molecules play a critical role in mediating the increase in adhesion (Duband et al., 1987; Horikawa et al., 1999; Linask et al., 1998; Radice et al., 1997). Cadherins are integral membrane glycoproteins expressed on the cell surface. They consist of a Ca^{2+}-binding extracellular domain containing a variable number of 110 amino acid repeats, a transmembrane domain, and a cytoplasmic domain (Koch et al., 1999). Cadherins cluster on the cell surface and bind to cadherins on adjacent cells



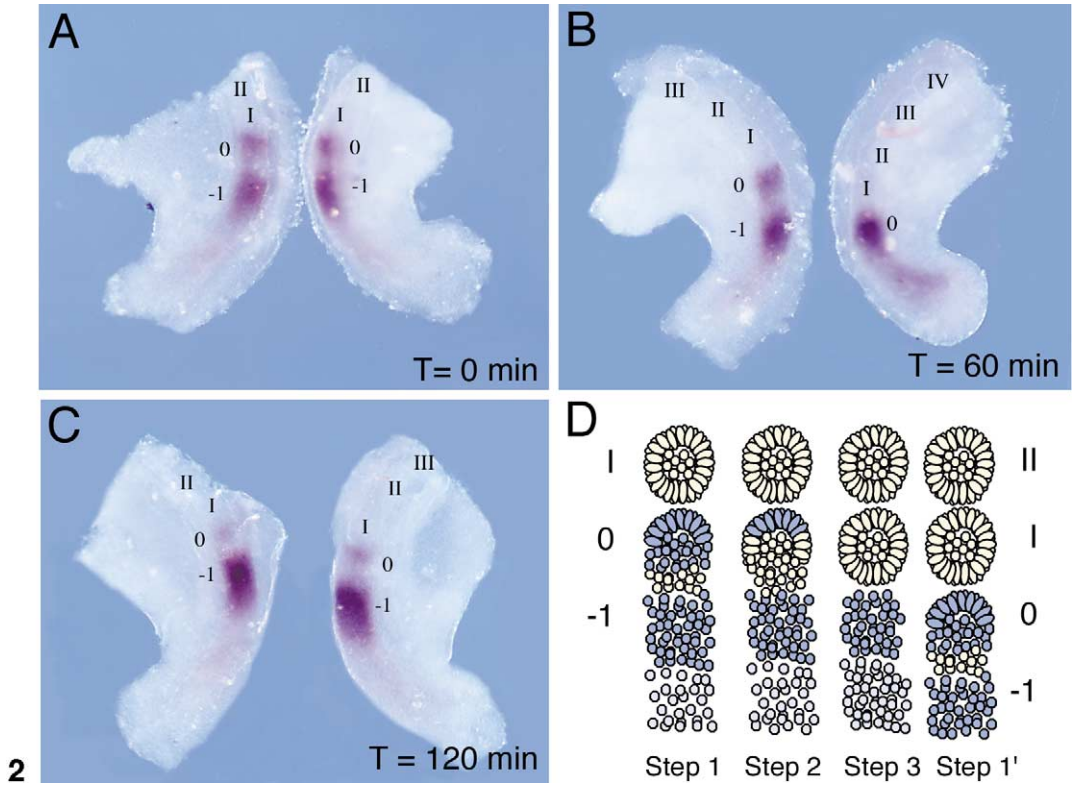
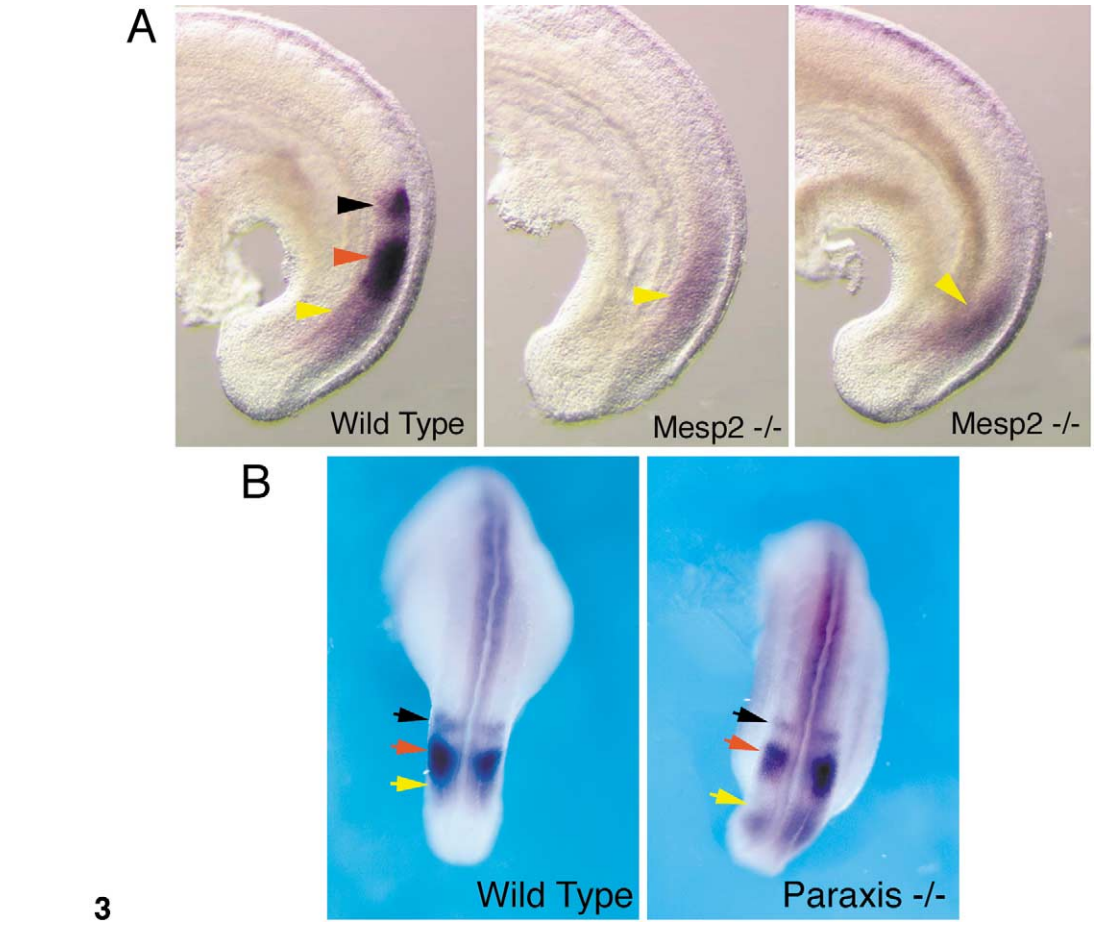


Fig. 1. The pattern of *papc* transcription is variable in the anterior presomitic mesoderm of the mouse embryo. *Papc* transcription is detected in the tail of 9.5 *dpc* mouse embryos by whole-mount in situ hybridization (A–C). The pattern of transcription varied between the width of transcription in somite 0 and the presence of transcription in somite—1 (A–C). Diffuse transcription of *papc* was constant in the posterior presomitic. The somites are numbered such that the newest somite is I and the forming somite is 0. Presumptive somites in the presomitic mesoderm are assigned negative integers. The site of the segmental boundary between somite I and 0 is marked with a black arrowhead. The transcription pattern of *papc* was compared to *Uncx4.1* in the contralateral halves of the embryo (D). *Papc* transcription was excluded from somite I, where *Uncx4.1* is transcribed.

Fig. 2. The pattern of *papc* transcription cycles over a 2-h period. The tail of 9.5 *dpc* embryos were disected sagittally. One-half of the embryo was fixed immediately and the other half was cultured for 0 h (A), 1 h (B), or 2 h (C). *Papc* transcripts were detected by whole-mount in situ hybridization. The two bands of transcription in the fixed half of the embryo had changed to a single band after 1 h (B). After 2 h, the pattern of *papc* transcription was the same as the fixed half except that it was shift posteriorly by a single somite (C). The transcription pattern of *papc* falls into three steps that can be superimposed on the cellular events associated with somite formation (D). Somites were numbered such that the newest somite is I and the next somite to be formed is 0.

through a Ca^{2+}-dependent homotypic interaction (Shapiro et al., 1995; Yap et al., 1997). The cytoplasmic domain interacts with β-catenin, which anchors the cadherins to F-actin and the cytoskeleton. β-catenin is also an integral component of the Wnt signal transduction pathway, raising the possibility that cadherins are able to modulate gene expression (reviewed in Gottardi and Gumbiner, 2001; Huber et al., 1996). N-cadherin is the primary cadherin associated with somitogenesis. N-cadherin is expressed in the anterior presomitic mesoderm and epithelial somites. Inhibition of N-cadherin with an antibody or by genetic inactivation disrupts the formation of epithelial somites (Duband



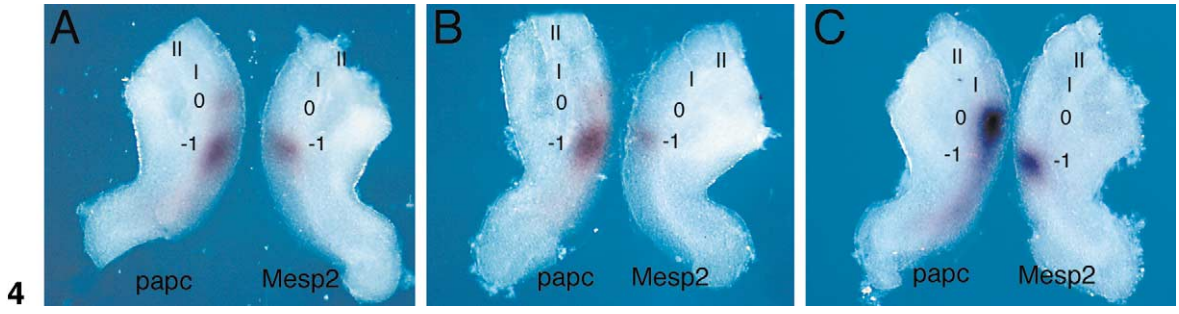


Fig. 3. *Papc* transcription is dependent on *Mesp2* in the anterior presomitic mesoderm. (A) Whole-mount in situ hybridization was performed on wild-type and *Mesp2*$^{-/-}$ 9.5 dpc embryos using a digoxigenin-labeled antisense RNA probe specific for *papc*. Three bands were observed in wild-type embryos that were consistent with somite 0 (black arrowhead), somite -1 (red arrowhead), and posterior presomitic mesoderm (yellow arrowhead). Only the posterior presomitic mesoderm expression was detectable in the *Mesp2*$^{-/-}$ embryos. (B) Transcription of *papc* did not vary between wild-type and *paraxis*$^{-/-}$ 9.5 *dpc* embryos.

Fig. 4. *Papc* is transcribed in an overlapping pattern with *Mesp2* in the presomitic mesoderm. The tail region of 9.5 *dpc* embryos was bisected along the midline and whole-mount in situ hybridization was performed with digoxigenin-labeled antisense RNA probes specific to *papc* and *Mesp2*. Three distinct combinations of transcription were observed. (A) *Mesp2* was transcribed broadly in somite -1, while *papc* is transcribed in the anterior half of somite 0 and broadly in somite -1. (B) The level of papc transcription is reduced in somite 0 at the time in which Mesp2 transcription narrows to the anterior half of somite -1. (C) In embryos where *papc* is transcribed in a broadband in somite 0 and not somite -1, *Mesp2* is transcribed in somite -1. Somites are numbered such that the newest somite is I and the next somite to form is somite 0.

et al., 1987; Linask et al., 1998; Radice et al., 1997). Somites deficient in *N-cadherin* coalesce into distinct anterior and posterior domains that can be distinguished by the transcription of the posterior-specific gene, *Uncx4.1*. This predicts a second level of cell-adhesion regulation that distinguishes the two halves of the somite (Horikawa et al., 1999).

Recent reports have identified the protocadherin, *papc*, another member of the cadherin superfamily, as an important regulator of somitogenesis during *Xenopus* develop-

ment (Kim et al., 2000). *Xenopus papc* (*xpapc*) is expressed in the anterior halves of the presumptive somites and anterior presomitic mesoderm, and its inactivation leads to the abnormal formation of somites. The mouse homolog of *xpapc* has been cloned and shown to be expressed in a banded pattern in the anterior presomitic mesoderm (Yamamoto et al., 2000). However, mice deficient for *papc* are viable and develop a normal skeletal system, suggesting that somitogenesis has not been disrupted.

Here, we describe an in-depth analysis of mouse *papc* transcriptional regulation in the anterior presomitic mesoderm. *Papc* is transcribed in a cyclical pattern in presumptive somite 0. *Papc* is initially expressed as a broad band that covers the entire anterior-to-posterior breadth of the presumptive somite and becomes restricted to the anterior half over the time required to make a single somite. During the somitic cycle, a second band of *papc* transcription, which is stable, is present throughout presumptive somite −1. Analysis of targeted null mutations revealed that *papc* transcription in this region is regulated by *Mesp2* and *L-fng*. Further, we inhibited the function of papc by treating mouse tail explant cultures with a secreted form of papc. This resulted in a failure of the presomitic mesoderm to epithelialize at the segmental border. Interestingly, an epithelium formed along the dorsal margin of the anterior presomitic mesoderm prior to overt somitogenesis and this occurred independently of papc. Therefore, we propose that *papc* is a morphogenic gene that is required for directing epithelialization at the site of border formation during somitogenesis.

Material and methods

Mice carrying targeted null mutations and genotyping

A targeted null mutation at the *paraxis* loci was described previously (Burgess et al., 1996). The genotype of the offspring of a *paraxis*$^{+/-}$ intercross was determined by Southern blot analysis by hybridizing *Sac*I-digested genomic DNA with a 300-bp genomic probe (Burgess et al., 1996). The *L-fng* mutant mice were described previously (Evrard et al., 1998); the presence of the mutant or wild-type allele of *L-fng* was determined by PCR, using a common 5′ primer [AGAGTTCCTGAAGC GAGAG] and a wild-type 3′ primer [GAG CACCAGGAGACAAGCC] or a PGKneo-specific 3′ primer [CTTGTGTAGCGCCAAG TGC]. A targeted null mutation at the *Mesp2* locus was described by Saga et al. (1997) and the genotyping procedure was performed as described in the reference.

Tail explant cultures

Dissection and culturing of mouse embryo tails was performed as described by Correia and Conlon (2000). Embryonic day 9.5 and 10.5 mouse embryos were dissected in cold dissection buffer [5.8 g/L NaCl, 0.186 g/L KCl, 0.05 g/L KH_2PO_4, 0.05 g/L $MgSO_4{\cdot}H_2O$, 0.0004 g/L $Na_2EDTA.2H_2O$, 0.336 g/L $NaHCO_3$, 0.25 g/L $CaCl_2$, 4.76 g/L Hepes (pH 7.1), 50 units/ml penicillin, 50 μg/ml streptomycin] supplemented with 10% fetal calf serum (FCS). The tail was separated from the remainder of the embryo between the third and fourth somite using drawn glass capillary tubes. In some experiments, the tail was cut longitudinally along the neural ectoderm and tail bud prior to culturing. Explants were cultured in 5% CO_2 atmosphere at 37°C, in 40 μl hanging drops of 1:1 Ham's F- 12:Dulbecco's modified Eagle's medium (DMEM) high-glucose supplemented with 10 ng/ml bFGF (Gibco-BRL, Gaithersburg, MD), 1:100 dilution of insulin-transferrin-selinium (ITS) (BioWhittaker, Walkersville, MD), and 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FCS for the time specified in the text.

In situ hybridization

Embryonic tissue was fixed in 10% neutral buffered formalin overnight at 4°C, washed briefly in PBS, and processed for whole-mount in situ hybridization as described by Wilson-Rawls et al. (1999). Tissue was hybridized with digoxigenin-labeled antisense RNA probes specific to *papc* (Yamamoto et al., 2000). *Uncx4.1* (Mansouri et al., 1997), *L-Fng* (Evrard et al., 1998), and *Mesp2* (Saga et al., 1997). All probes were generated as described by Wilson-Rawls et al. (1999).

Cloning and production of pape-AP

A secretable pape–alkaline phosphatase fusion protein was generated by cloning the extracellular domain of papc into APtag-5 vector (GenHunter Co., Nashville, TN) in-frame with the Ig kappa-chain secretion signal and alkaline phosphatase. *Papc* cDNA that contains the repeating cadherin repeats was generated by RT/PCR using total RNA from 9.5 *days postcoitum* (*dpc*) and *papc*-specific amplimers (5′ primer—CCAGCCGG CCAAGACAGTCCGATACAGC and 3′ primer—CCTCCGGACCCCGACGGCGCGA GGCG). The papc–AP fusion protein was produced as described by Flanagan et al. (2000). Papc–AP plasmid was transiently transfected into 293T cells using Lipofectamine and PLUS reagent (Invitrogen, Carlsbad, CA). Since the parent vector contains an SV40 origin of replication, Papc-AP is maintained at a high copy number in 293T cells that express SV40 large T antigen. Cells were cultured for 3 to 5 days in DMEM (BioWhittaker), supplemented with 10% fetal bovine serum (Gemini BIO-PRODUCTS) and penicillin (50 IU/ml) and streptomycin (50 IU/ml) (Mediatech, Inc., Herndon, VA). Supernatant was collected; contaminating cells were removed by low speed centrifugation and then stored at 4°C. The fusion protein was concentrated using the 10K Macrosep MF filtration system (Pall Life Sciences) and dialyzed against 1 L of 1:1 DME/F12 overnight at 4°C. The concentration of the fusion protein was determine using BIAcore biosensor system with an anti-His

mAb (Sigma) immobilized on a rabbit anti-mouse Cm5 chip as described previously (Young et al., 2002).

To visualize binding of the papc–AP fusion protein to the presomitic mesoderm, dissected embryos were incubated overnight in 2–5 ml of supernatant from 293T transfected cells at 4°C with rocking. Embryos were washed with 2 ml of Hanks' balanced salt solution (HBSS) 10 times for 5 min followed by an overnight wash at 4°C. Embryos were fixed in 10% neutral buffered formalin for 10 min at room temperature followed by three washes with HBSS. Endogenous alkaline phosphatase activity was inactivated by incubation at 61°C in HBSS overnight. The following day, embryos were washed twice in CT.3 [100 mM Tris–Cl (pH 9.5), 150 mM NaCl, 25 mM $MgCl_2$, 0.3% Triton X-100] for 15 min and incubated 1 to 3 days in BM Purple AP Substrate (Roche, Basel, Switzerland). The color reaction was stopped by rinsing in 10 mM Tris–Cl (pH 9.0), 1 mM EDTA for 20 min, followed by fixation in 10% formalin overnight.

Disruption of papc-mediated cell adhesion was performed by culturing 9.5 *dpc* tails for 4 h in 1:1 Ham's F-12:DMEM high glucose supplemented with 10 ng/ml bFGF (Gibco-BRL), 1:100 dilution of ITS (BioWhittaker), and 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum containing ~50 pg/μl of the papc–AP fusion protein. Secreted AP was used as a control in all experiments. Morphological changes were examined in the anterior presomitic mesoderm by staining the tissue with Alexa Fluor 488 phalloidin (Molecular Probes), an actin-binding compound. Tissue was fixed in 100% acetone for 3 min and then rehydrated with PBS. Tissue was stained overnight in the dark with a 1:40 dilution of Alexa Fluor 488 phalloidin in PBS. After incubation, samples were washed five times with PBS. Phalloidin staining was visualized using a Leica NTS confocal microscope equipped with an argon laser.

Immunohistochemistry

To perform immunohistochemistry on the embryonic tails, tissue was fixed overnight in 10% neutral buffered formalin and then washed thoroughly with PBS. The tissue was incubated for 1 h at room temperature (RT) in ICB [20 mM HEPES, 5 mM EGTA, 5 mM $MgCl_2$, 100 mM KCl, pH 6.8] supplemented with 5% nonfat dry milk and 0.5% Tween- 20. This was followed by an overnight incubation at 4°C in ICB buffer containing 1% nonfat dry milk, 0.1% Tween-20, 20% normalized goat serum (Jackson ImmunoResearch Laboratories), and a 1:250 dilution of antilaminin antibody (Sigma, Cat. No. L 9393). The tissue was washed six times at room temperature with the same buffer without antibody. This was followed by incubation overnight at 4°C with the secondary antibody diluted 1:250 (Molecular Probes, Cat. No. A-11011) in the same buffer. The tissue was washed three times and then placed in PBS until imaging. For double labeling for actin, the samples were treated with 30, 60, and 100% acetone for 1 min each. Then, the samples were rehydrated and washed in PBS. Samples were placed in Alexa Fluor 488 conjugated phalloidin (Molecular Probes, Cat. No. A-12379) diluted 1:40 and incubated overnight at RT. Samples were washed twice in PBS and mounted for visualization by confocal microscopy.

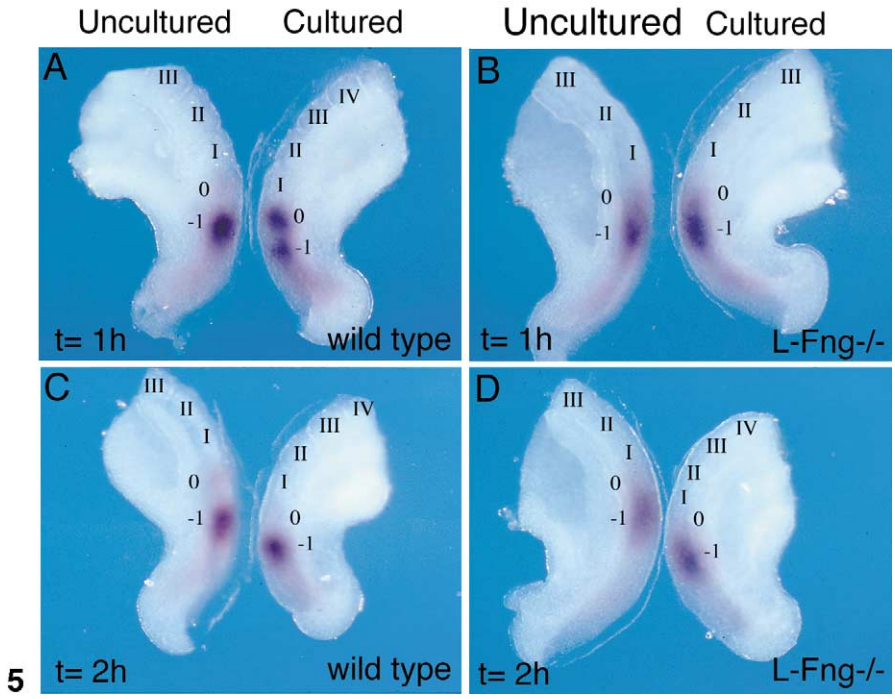
Results

Papc is transcribed in oscillating pattern in the first somite

Papc is transcribed in three distinct domains of the presomitic mesoderm of the developing mouse embryo (Yamamoto et al., 2000). At 9.0 dpc, transcription was restricted to two stripes at the anterior end of the presomitic mesoderm that are consistent with the next two presumptive somites (somite 0 and −1) and a third diffuse signal in the posterior region of the presomitic mesoderm (Figs. 1A–1C). A comparison of *papc* transcription in several embryos revealed variations in the spatial distribution of RNA in the anterior-most band that ranged from a broad band to a narrow band at the anterior edge (Figs. 1A and 1B, see arrowhead). In other embryos, a single broadband was observed in the anterior presomitic mesoderm.

To determine whether *papc* transcription was in the forming somite (0) or the newest somite (I), the transcription of *papc* was compared to *Uncx4.1,* a transcription factor expressed in somite I, but excluded from somite 0 (Mansouri et al., 1997). When embryos were dissected sagittally and the two halves were examined for either *papc* or *Uncx4.1* transcription by whole-mount in situ hybridization, no overlap was observed (Fig. 1D). This indicated that *papc* transcription was limited to the presomitic mesoderm.

The observed transcription of *papc* is similar to the oscillating patterns observed for genes that are regulated by the segmentation clock in the presomitic mesoderm. To determine if the *papc* transcription pattern cycled within individual embryos, the tails of 9.0 dpc embryos (transected between somite III and IV) were isolated and cut sagittally along the neural tube. One half of each embryo was immediately fixed, while the other half was cultured under conditions that promoted somitogenesis (Correia and Conlon, 2000). Somites formed in culture approximately every 2 h, similar to what has been observed in utero (data not shown). Tails were cultured for 1 or 2 h, followed by detection of *papc* mRNA by whole-mount in situ hybridization. In a control embryo, where both sides were fixed immediately, the pattern of transcription did not vary (Fig. 2A). However, after 1 h of culturing, a single band was present in somite 0 instead of the two bands of papc transcripts in the uncultured half (Fig. 2B). Embryonic halves cultured for 2 h had the same pattern of papc transcription as the uncultured side, except that it was shifted posteriorly by a single somite (Fig.



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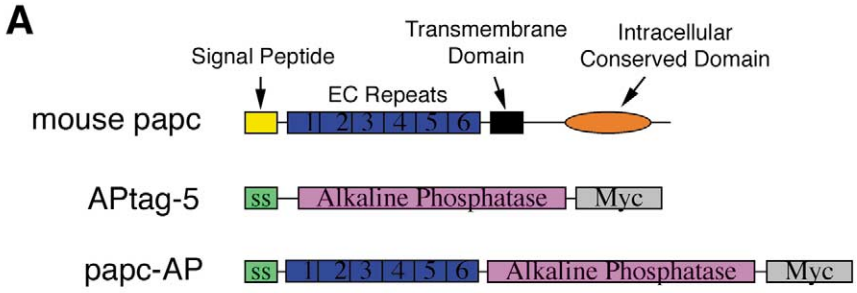

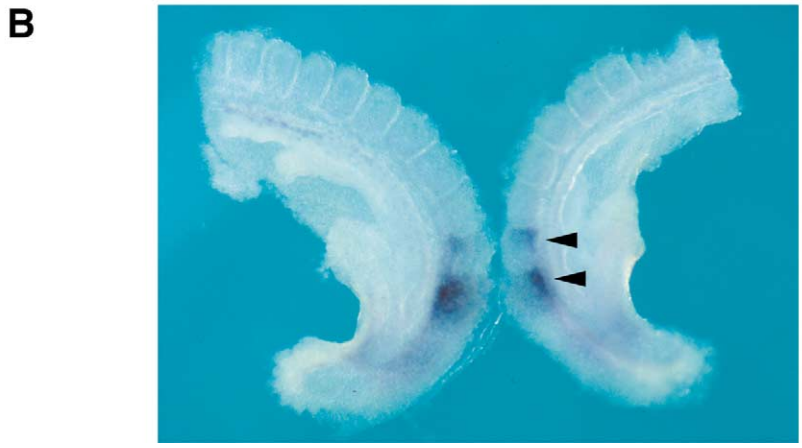

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Fig. 5. The dynamic transcription of *papc* in somite 0 is lost in the absence of *L- Fng*. The tail region of wild-type (A, C) and *L-fng*$^{-/-}$ (B, D) 9.5 *dpc* embryos were bisected along the midline and one-half was cultured for 1 h (A, B) or 2 h (C, D). Whole-mount in situ hybridization was performed with a digoxigenin-labeled antisense RNA specific to *papc*. In the absence of *L-fng*, only one band was observed in the anterior presomitic mesoderm. After 1 h of culture, no difference was observed in the pattern of *papc* transcription in the mutant embryos. The pattern of transcription in the mutant embryo had shifted posteriorly after 2 h, similar to what was observed in the wild-type embryos.

Fig. 6. A secreted form of papc fused to alkaline phosphatase bind specifically to paraxial mesoderm. (A) Mouse *papc* contains six extracellular cadherin (EC) repeats, a signal peptide, transmembrane domain, and a conserved intracellular domain. The EC repeats of mouse *papc* was cloned in-frame into Aptag-5 between an Ig kappa secretory signal and a sequence coding for alkaline phosphatase. (B) Wild-type mouse embryos were treated with conditioned media containing papc–AP. Binding was visualized by a colorimetric assay for alkaline phosphatase. The tail region of the embryo was bisected and the neural tube removed to allow visualization of the pattern in the presomitic mesoderm and somites. The two strong bands near the anterior presomitic mesoderm (black arrowheads) and fainter diffuse band in the posterior presomitic mesoderm are similar to what was observed for *papc* transcripts. In some embryos, the narrower anterior band is in the newly formed somite.

2C). These data demonstrate that *papc* transcription is cycling over a 2-h period. We propose that *papc* transcription can be divided into three distinct steps (Fig. 2D). In the first step, *papc* is transcribed broadly in both somite 0 and −1. Transcription is progressively restricted in an anterior direction in somite 0 during step 2. In the final step, at the time that the segmental boundary was formed, *papc* transcription is lost in these cells. The broadband in somite −1 is now reassigned to somite 0. When *papc* transcription is induced in the new somite −1, the cycle has returned to step 1.

Papc transcription is regulated by Mesp2

Specification of cells to the anterior lineage in somites is dependent on the expression of *Mesp2* in presumptive somite −1 (Saga et al., 1997; Takahashi et al., 2000). Since the transcription of *papc* becomes restricted to the anterior half of somite 0, we examined whether *Mesp2* regulated *papc* transcription. In 9.5 dpc *Mesp2*$^{-/-}$ embryos shown in Fig. 3A, *papc* RNA was absent in the anterior presomitic mesoderm, but was expressed in the posterior presomitic mesoderm and neural tube. This indicated that *Mesp2* is required for the transcription of *papc* in the anterior presomitic mesoderm. The continued transcription in the posterior presomitic mesoderm points to regulation of *papc* by other factors in this region.

The transcription factor *paraxis* is also required for the restricted transcription of genes in the anterior and posterior halves of the somite (Johnson et al., 2001). It has been proposed that paraxis acts downstream of *Mesp2* and the specification of cells to the anterior region by restricting the movement of cells across the A/P boundary. Papc is a candidate molecule for participating in the restriction of cell movement because it is a cell adhesion molecule. To determine whether *papc* was regulated by *paraxis*, its expression was examined in 9.5 dpc *paraxis*$^{-/-}$ embryos (Fig. 3B). When compared to wild-type littermates, no difference was observed in the expression pattern of *papc*, thus *paraxis* is not required.

A comparison of *papc* and *Mesp2* transcription patterns revealed overlap in the region of somite −1 (Fig. 4). The tails of wild-type 9.5 dpc embryos were split sagittally and probed by whole-mount in situ hybridization for *Mesp2* or *papc* mRNA. In the majority of the embryos analyzed, *papc* transcripts were present in somite 0 and −1, as described above. In these embryos *Mesp2* transcripts were present in a broadband that overlapped with *papc* message in somite −1 (Fig. 4A). When *Mesp2* transcription became restricted to the anterior half of somite −1, *papc* transcription is faint in somite 0 and unchanged in somite −1 (Fig. 4B). Immediately after somite formation, when *papc* was present as a single band in somite 0, *Mesp2* was already transcribed in the new somite −1 (Fig. 4C). This indicates that *Mesp2* transcription precedes *papc* transcription in somite −1, consistent with a role in regulating its transcription. However, *Mesp2* is not actively transcribed in somite 0, raising the possibility that other genes are required to maintain *papc* in these cells.

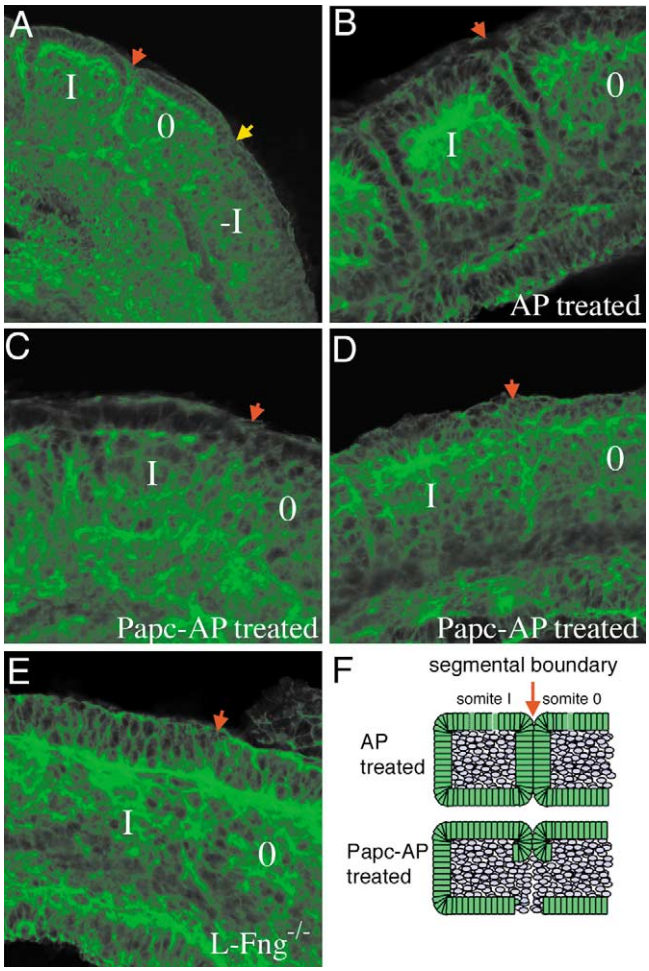

Fig. 7. Papc–AP treatment disrupts the formation of the epithelium at the segmental border. The expression of the F-actin was visualized in the presomitic mesoderm and somites of a 9.5 *dpc* embryo by staining with Alexa Fluor 488 phalloidin (A). The effect of culturing the tails of 9.5 *dpc* embryos in the presence of AP (B) or papc–AP fusion protein (C and D). In both treatments, the epithelium can be distinguished along the dorsal edge of the somites and anterior presomitic mesoderm based on cell density and organization of actin staining along the ventral surface. (B) In AP-only treated embryos, a clear segmental boundary formed between epithelial cell layers of somite I and O (red arrows). A second boundary is forming between somite I and the forming somite in the presomitic mesoderm (yellow arrows). The epithelium on either side of the boundary between somites I and O fail to extend ventrally in embryos treated with papc–AP (C and D). In the *L-fng*$^{-/-}$ mice (E), the segmental boundary is absent similar to the papc–AP-treated embryos. A schematic of the papc–AP induced defect in epithelialization at the segmental boundary (F).

Regulation of papc by segmentation clock

Papc transcription in somite 0 cycles at the same frequency as the segmentation clock, suggesting that the two may be linked. The Notch signaling pathway plays an integral role in translating the temporal periodicity of the segmentation clock into segmentation borders. Inactivation of

L-Fng results in a loss in the oscillation of gene expression associated with the segmentation clock (Aulchla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998). To determine whether *papc* transcription is linked to the segmentation clock, *papc* mRNA was examined in 9.5 dpc *L-Fng*$^{-/-}$ and wild-type embryos. In uncultured tissue, a single broadband was consistently observed in the anterior presomitic mesoderm of the *L-Fng*$^{-/-}$ embryos (Figs. 5B and 5D). To determine the influence of *L-Fng* on the cycling of *papc* transcription in somite 0, one-half of the tail was cultured for either 1 or 2 h. After 1 h there was no difference in the pattern of *papc* transcription (Fig. 5B). However, the position and breadth of the *papc* RNA domain was similar to the uncultured half after 2 h except that it was shifted posteriorly (Fig. 5D). Together, these patterns are consistent with a failure of *papc* transcription to undergo the progressive restriction to the anterior domain of somite 0. It is interesting to note that the posterior shift in *papc* transcription occurs at the same frequency as the wild-type embryo and thus is independent of *L-Fng* (Fig. 5C).

Disruption of papc activity in the presomitic mesoderm

The oscillating transcription pattern of *papc* in the anterior presomitic mesoderm and its regulation by *Mesp2* and *L- Fng* predicts that this gene plays a role in regulating somite formation. To examine the function of papc during somitogenesis, a soluble form of the gene product was generated that could potentially act as a dominant negative mutant. Since protocadherins interact in a Ca^{2+}-dependent homophilic manner (Obata et al., 1995), a high concentration of the secreted form of the protein will interact preferentially with papc on the cell surface, thus disrupting the cell-adhesion properties of the protein in the tissue. A similar approach has been used to disrupt papc activity in *Xenopus* (Kim et al., 2000). For our studies in mice, a secretable form of mouse papc was generated that contains the six extracellular cadherin domain repeats (+104 to +2219 nt) fused to alkaline phosphatase (AP) (Fig. 6A). The *papc–AP* gene also contains the Ig kappa-chain secretion signal to allow for secretion of the protein in transfected 293T cells.

To demonstrate the ability of the fusion protein to bind specifically to endogenous papc molecules, 9.5 dpc mouse embryos were incubated in the supernatant of 293T cells transfected with a plasmid that constitutively expresses either the *papc-AP* or the *AP* genes. The sites of protein interaction were visualized by incubating the embryo in a colorimetric substrate for alkaline phosphatase. Papc–AP binding was readily detectable in two bands in the region of the anterior presomitic mesoderm that is consistent with the transcription pattern of *papc* (Fig. 6B). When the tail region was bisected and the neural tube removed, it was clear that the binding of papc–AP in this region was specific for paraxial mesoderm. In some cases, binding was observed in the anterior half of the first somite, suggesting that the protein was maintained after transcription had stopped (Fig. 6B). Interestingly, binding was not observed in the CNS, where papc transcription has also been reported (Yamamoto et al., 2000). This may be due to the inaccessibility of this tissue to the fusion protein or that the level of detection is not sensitive enough. It is important to note that papc–AP did not bind in the broader pattern described for N-cadherin and cad11 expression. This demonstrates that the EC repeats of papc do not have a high affinity for the EC repeats of the cadherins expressed in the presomitic mesoderm and somites.

To examine the role of papc in regulating somitogenesis, 9.5 dpc tails were cultured in the presence of secreted papc–AP or AP alone for 4 h. Embryos were then fixed and stained with Alexa Fluor 488 phalloidin, which binds specifically to the F- actin component of the cytoskeleton. The cellular organization of the somites and presomitic mesoderm stained with phalloidin were visualized by confocal microscopy. The epithelial components of the newly formed somites could be distinguished from the mesenchymal somitocoel (Fig. 7A). Epithelial cells could be distinguished from mesenchymal cells based on their high density and foci of phalloidin staining the site of cytoskeletal condensation at the focal adhesion sites along the apical surface. Strong staining was also present along the basal surface of the somite epithelium at the boundaries between somites (Figs. 7A and 7B, red arrows). Interestingly, cells organized into an epithelium extend to somite 0 on the dorsal side of the anterior presomitic mesoderm (Fig. 7A). This indicates that the epithelium along the dorsal side of the somite forms prior to segmentation and independently of epithelium at the segmental border. A similar pattern of epithelialization has been observed in the avian presomitic mesoderm but not in mouse (Palmeirim et al., 1998).

Culturing tail explants in the presence of the papc–AP fusion protein had a significant impact on the formation of the segmental boundary. In embryos that were treated with papc–AP, the epithelium at the segmental boundary failed to form properly (Figs. 7C and 7D). The site of the segmental boundaries (Fig. 7, red arrowheads) could be identified by the separation of the dorsal epithelium. However, the epithelium along the segmental border was truncated ventrally. In control embryos treated with AP-conditioned medium, the newly formed somite possessed the normal organization, including a clear segmental boundary between somites I and 0 and a distinguishable epithelium (Fig. 7B). This indicates that the disruption in epithelialization is due to the papc inhibitor. Interestingly, an examination of somites I and 0 from the *L-fng*$^{-/-}$ embryos revealed a similar phenotype (Fig. 7E). The dorsal epithelium formed a relatively continuous sheet with an absence of clear segmental boundaries between somites, predicting that the loss of Papc activity at the segmental boundary and ubiquitous expression have the same impact on epithelialization. This supports the hypothesis that differential activity of Papc at

the forming segmental boundary is crucial to epithelialization.

The basement membrane associated with the basal surface of the epithelial somite contains laminin. Therefore, the pattern of laminin can be used as an alternative method of examining the epithelial state of the newly formed segmental boundary. Immunohistochemistry was performed on the AP- and papc–AP-treated embryos using an antibody specific to laminin. The tissue was also stained with phalloidin to allow for the visualization of the stress fibers in the somites. In the AP-treated embryo, laminin clearly presents along the dorsal and ventral sides of the somite and clearly demarcates the segmental boundary between somites I and 0 (Fig. 8C). The organization of the laminin along the future segmental boundary can be seen in the ventral region of the presomitic mesoderm. An overlay of the phalloidin and antilaminin stain demonstrate that the strong actin stain that extends from the dorsal to ventral surfaces coincides with the laminin expression, supporting the conclusion that this is the segmental boundary (Fig. 8E). Consistent with the disrupted pattern of actin in the papc–AP-treated embryos, laminin is absent along the predicted segmental boundary (Fig. 8D, red arrows). Some laminin expression was present along the ventral side of the somite; however, the signal was diffuse. Therefore, the laminin expression pattern supports the conclusion that inhibition of papc disrupts epithelialization at the segmental boundary.

Discussion

Somitogenesis is a dynamic process that is regulated by tightly linked spatial and temporal events. Cells at the anterior end of the presomitic mesoderm are separated from the adjacent cells by the formation of a segmental boundary, while adopting an epithelial morphology. The positional identity of the cells, which has been established along the A/P axis, must be also maintained. A central issue in somitogenesis is gaining a clear understanding of the genetic link between the segmentation clock and the morphological events associated with somite formation. In studies performed in zebrafish and *Xenopus* it has been demonstrated that *papc* is regulated in an anterior-specific manner in the somite. Disruption of *papc* in these systems results in abnormal patterning of the forming somite (Kim et al., 2000; Sawada et al., 2000). Here we demonstrate that mouse *papc* is also required for mouse somitogenesis. *Papc* is required for the morphological transition of mesenchyme at the segmental boundary to epithelium. Transcription of *papc* is dependent on both *Mesp2,* which specifies the anterior domain of the somite, and *L-Fng,* which regulates the segmentation clock. This indicates that the transcription of the mouse *papc* lies at the nexus between the two regulatory pathways linking the segmentation clock to morphological events associated with segmentation.

Regulation of papc transcription by genes that control somite segmentation

Analysis of *Mesp2*$^{-/-}$ embryos revealed that this gene is required for transcription of *papc* in somites 0 and −1. Similarly, it has been demonstrated that *Xenopus* and zebrafish homologs of *papc* are also regulated by *Mesp2,* indicating that this pathway is evolutionarily conserved (Kim et al., 2000; Sawada et al., 2000). Inactivation of *Mesp2* results in a failure to form distinct segmental boundaries, including a loss of epithelialization at the boundary (Saga et al., 1997; Sawada et al., 2000; Sparrow et al., 1998; Takahasi et al., 2000). Our studies demonstrate that *papc* is important for organizing epithelial cells along the segmental boundary. This suggests that the *Mesp2*-directed epithelialization is mediated in part through the expression of *papc.* In support of this hypothesis, an analysis of a hypomorphic allele of mouse *Mesp2* revealed that the transcription of *papc* correlated with the formation of segmental boundaries (Nomura-Kitabayashi et al., 2002).

The dynamic nature of *papc* transcription in somite 0 in the mouse differs from the static expression patterns reported in *Xenopus* and zebrafish. The importance of this transcription pattern is not fully understood. It is possible that differential expression of *papc* at the border between putative somites 0 and −1 is important for organizing cells when the segmental boundary forms at this site. It has been demonstrated that differences in the repertoire of cell adhesion molecules expressed on cells at a cellular boundary can lead to preferential cell sorting (Dahmann and Basler, 1999). Differential expression of cadherin subtypes has been demonstrated to be important for compartmentalization of developing organs, including the heart and CNS (Inouc et al., 1997, 2001; Linask et al., 1997; Redies, 2000). Thus the expression of papc on the posterior side of the border could promote the organization of cells along the border. Thus the absence and overexpression of *papc* both would result in a disruption in the organization of the cells at the border. This model is consistent with the observation that *L-Fng*$^{-/-}$ embryos, which fail to downregulate *papc* transcription in the posterior half of somite 0, also fail to form a distinct epithelium at segmental borders.

The abnormal pattern of *papc* mRNA in the *L-Fng*$^{-/-}$ embryos suggests that transcription of the gene in somite 0 is inhibited by activation of the Notch signaling pathway. It remains to be determined whether members of *Hes* or *HesR* family of bHLH transcription factors, which are upregulated by Notch activation, directly regulate *papc* transcription. Members of the *Hes* family have been shown to act as transcriptional repressors. Alternatively, Notch could repress *papc* transcription indirectly. It has been reported that Notch is able to repress *Mesp2* transcription in the posterior half of somite −1 (Koizumi et al., 2001). It is possible that the loss of *papc* transcription is due to reduction of Mesp2 protein in the posterior half of the presumptive somite.

The observation that blocking papc-mediated cell adhe-

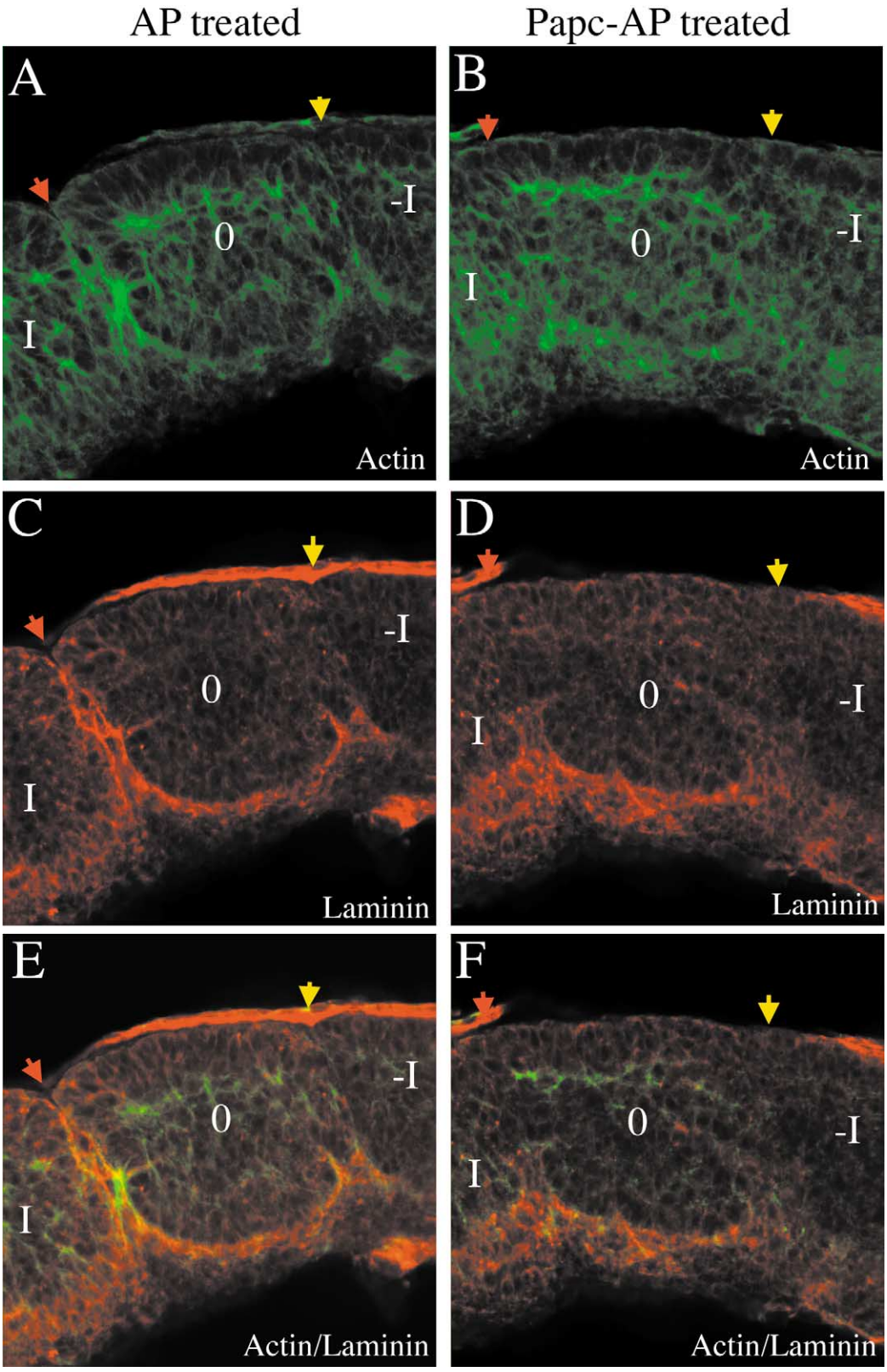

Fig. 8. Laminin expression was absent at the segmental boundaries in papc–AP-treated embryos. AP (A, C, E) and papc–AP (B, D, F)-treated 9.5 *dpc* embryos were stained for F-actin (A and B) using Alexa Fluor 488 phalloidin and for laminin (C and D). The newly formed segmental boundaries are demarcated with a red arrow and the forming boundary is demarcated with a yellow arrow. Regions of coexpression of actin and laminin are visualized by overlaying the images (E and F).

sion disrupts normal somitogenesis seems inconsistent with the report that *papc*$^{-/-}$ mice develop normally (Yamamoto et al., 2000). Protocadherins are a large subfamily of the cadherin superfamily with greater than 60 members; these proteins are characterized by six or more extracellular cadherin repeats (Wu et al., 2001). It has been proposed that multiple protocadherins that are functionally redundant might be expressed in the anterior presomitic mesoderm. Therefore, the secreted papc–AP form of the protein could inhibit the interaction of additional members of the protocadherin family. However, no other protocadherins have been reported that have the same expression pattern as *papc* in the presomitic mesoderm. Alternatively, the addition of the soluble papc–AP protein may produce a different response in the presomitic mesoderm than the targeted null mutation. The homotypic interactions of members of the cadherin superfamily elicit an increase in intracellular association with the cytoskeleton. This is mediated through the binding of proteins such as β-catenin, plakoglobin, and plakophlin-1 to the intracellular domain of the cadherins. It has been reported that members of the protocadherin subfamily are able to interact with two genes that participate in signal transduction pathways, Fyn, a tyrosine kinase receptor belonging to the Src family (Kohmura et al., 1998), and reelin (Senzaki et al., 1999). Thus, it is possible that the addition of the papc–AP fusion protein is able to disrupt cell–cell adhesion while still activating the intracellular signaling pathway. This would produce a different response from the inactivation of the gene. Finally, the biological significance of disrupting the epithelium at the segmental border is not known. It is possible that this morphological defect does not result in any change in the patterning and differentiation of cartilage and muscle that are generated from the somite.

Model of papc-mediated segmentation

The analysis of the papc–AP-treated embyros using the Phalloidin stain provided insight into the mechanism by which mesenchymal cells in somite 0 adopt an epithelial fate. Epithelialization along the dorsal surface appears to occur independently of the epithelium at the segmental border. This is consistent with the phenotype of the *Mesp2*$^{-/-}$ embryos, where the segmental boundaries are disrupted but not the lateral epithelium (Saga et al., 1997). This suggests that the epithelium that traverses the presomitic mesoderm requires an additional level of regulation from the epithelium located around the perimeter. The phenotype of the papc–AP-treated tail explants is strikingly similar to the phenotype of the *Mesp2*$^{-/-}$ embryos, indicating that *Mesp2* directs epithelialization at the boundary by the induction of *papc* transcription. It is not clear from our studies whether papc is also involved in forming the segmental boundary. It has been demonstrated with the *paraxis*$^{-/-}$ embryos that segmentation is not dependent on epithelialization (Burgess et al., 1996). In addition, the *papc*$^{-/-}$ display no clear segmentation defect. However, the differential expression of *papc* along the segmental border and its regulation by the Notch signaling pathway are suggestive of a role in segmentation.

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